TGF-β-Induced (TGFBI) Protein in Melanoma: A Signature of High Metastatic Potential

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Tumor-produced extracellular matrix (ECM) proteins can be key elements in tumor growth and metastasis. Transforming growth factor beta–inducible (TGFBI) protein is a secreted ECM component that can have dual function in cancer, acting as tumor suppressor or promoter. Although TGFBI is expressed in human melanoma cells, the exact role it might have in melanoma metastasis remains elusive. Assessing the expression and secretion of TGFBI, we show that human metastatic melanomas express and secrete significantly higher amounts of TGFBI, compared with nevus lesions and primary melanoma tumors. Intravenous injection of highly metastatic human melanoma cells expressing shRNA that targets TGFBI assigns a critical role for TGFBI in the formation of melanoma distal metastases in nude mice. *In vivo* assays demonstrate that TGFBI silencing does not interfere with melanoma cells' dissemination to distal sites but rather with their proliferation and outgrowth within new microenvironment. In line, TGFBI silencing increases melanoma cells motility/invasion/extravasation *in vitro* but interferes with their progression through the cell cycle, drastically reducing their proliferation. Furthermore, we show that TGFBI is a regulator of cyclins and cyclin-dependent kinases in melanoma. Collectively, our data describe a mechanism of melanoma metastatic outgrowth via promotion of growth/survival by the ECM protein TGFBI.

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INTRODUCTION

Melanomas are highly aggressive malignancies that frequently disseminate, remaining therefore an enormous clinical challenge. The factors involved in their metastatic outgrowth are not yet fully explored, and a deeper understanding of such factors should lead to more effective therapies (Spano *et al.*, 2012).

The extracellular matrix (ECM) proteins, compromising tumor microenvironment, can be key elements in tumor growth and metastasis (Wong and Rustgi, 2013). They can act as regulators of survival, proliferation, differentiation, and cell migration/invasion. Melanoma cells often modify their microenvironment through secretion of specific ECM proteins and ECM-modifying proteases to be more permissive for their growth and dissemination (Botti *et al.*, 2012).

The transforming growth factor beta (TGFβ)-induced protein (TGFBI) is a secreted ECM protein mainly expressed in fibroblasts, keratinocytes, and muscle cells. It can associate with several ECM proteins and function in many cell types as a linker protein connecting various matrix molecules to each other facilitating cell–ECM interactions (Thapa *et al.*, 2007).

In cancer, the expression and functional role of TGFBI has been controversial. TGFBI was found increased in various tumors (Sasaki *et al.*, 2002; Ma *et al.*, 2008; Lin *et al.*, 2010; Turtoi *et al.*, 2011; Ma *et al.*, 2012) whereas downregulated in others (Kang *et al.*, 2010; Kaiser *et al.*, 2013). However, recent findings in ovarian cancer have indicated that TGFBI could probably has dual function acting as tumor suppressor or promoter (Ween *et al.*, 2012). In melanoma, while microarray analysis showed increased expression of TGFBI in cells from metastatic and vertical growth phases (Hoek, 2007; Nummela *et al.*, 2012), TGFBI was also identified as decitabine-induced gene through epigenetic histone modification (Halaban *et al.*, 2009). We found that TGFBI is upregulated in multicellular melanoma spheroids, which possess high metastatic potential (Ramgolam *et al.*, 2011). These studies have been supported by recent findings showing that TGFBI is located in fibrillar structures in human melanoma metastases, and knockdown

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Abbreviations: CCN, cyclin; CDK, cyclin-dependent kinase; ECM, extracellular matrix; GFP, green fluorescent protein; TGFBI, transforming growth factor β–induced protein; TGFβ, transforming growth factor β; WT, wild type

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of TGFBI expression in melanoma cells impaired tumor formation and vertical growth phase (Nummela *et al.*, 2012). However, the exact role of TGFBI in melanoma metastasis remains incompletely understood.

In this study, we developed a metastasis model based on intravenous injection of highly metastatic 1205Lu human melanoma in nude mice and assessed the effect of silencing TGFBI on melanoma cells' dissemination and metastases formation. Our results show that lack of TGFBI secretion by metastatic melanomas results in drastic reduction in their distal metastatic outgrowth mainly by disturbing their cell cycle progression likely with defects in G1/S transition and S phase regulation.

RESULTS

Metastatic melanomas produce large amounts of TGFBI

We first compared the mRNA expression in biopsies from human nevi lesions, primary skin and metastatic melanomas. Primary skin melanomas showed little variations in *TGFBI* level compared with benign nevi (1- to 2-fold induction). *TGFBI* expression levels were higher in metastatic melanomas than in benign lesions or primary melanomas (ranging from 1.5- to 9.5-fold induction) (Figure 1a). This scattered variation in *TGFBI* mRNA expression was not tissue specific, but we could clearly define two groups of metastatic melanomas, a low (1- to 2.5-fold induction) and a high (2.7- to 9.8-fold induction) expressing group. To see the potential relevance of these findings, we then performed TGFBI immunostaining in human nevi lesions, primary melanomas, and skin, liver and lymph node metastases. Despite heterogeneity in the expression level, nevi lesions showed non- or very faint TGFBI staining (Figure 1b). In contrast, TGFBI was detected around melanoma cells in primary and metastatic lesions (Figure 1b), which is in accordance with being a secreted ECM and with its reported localization within fibrillar structures surrounding melanoma cells (Nummela *et al.*, 2012). Compared with nevus lesions and primary tumors, TGFBI staining was more intense in metastases, suggesting that metastatic melanoma cells secrete higher amounts of TGFBI than primary melanomas.

To verify the possibility that TGFBI was secreted by melanoma cells and not surrounding cells, we compared the secretion of TGFBI in different cell lines derived from human primary melanomas (WM983A and WM793) or their respective metastatic counterparts from the same patients (WM983B, 1205Lu). All melanoma cell lines secrete large amounts of TGFBI, but secretion levels were significantly higher in metastatic ones (Figure 1c).

TGFBI is required for melanoma metastatic outgrowth

The above findings suggest that the secretion of TGFBI by melanoma cells might have a role in melanoma metastasis, therefore we developed a metastasis model and assessed the effect of silencing TGFBI. Metastatic melanoma cells were engineered to express either a non-target shRNA (CTRL-GFP (green fluorescent protein)-shRNA-1205Lu) or shRNA that targets TGFBI (GFP-TGFBI-shRNA-1205Lu) (Supplementary Figure S1 online) and were injected into the retro-orbital venous sinus of immunodeficient mice. All mice that received TGFBI-producing CTRL-shRNA-1205Lu cells (16 mice) showed significant weight loss 3 weeks post injection (Figure 2a). Three out of the 16 recipient mice died very early (day 15), and the remaining mice were moribund 15–47 days post injection (Figure 2b). In contrast, mice that received TGFBI-shRNA-1205Lu cells (15 mice) producing very low levels of TGFBI (Supplementary Figure S1b and c online) did not show major changes of their weight (Figure 2a), showed delay in disease development, and increase in disease-free survival (6/15 TGFBI-shRNA-1205Lu challenged mice) (Figure 2b).

CTRL-shRNA-1205Lu group (10/10) developed proximal tumor metastases while only 9/15 mice that received TGFBI-shRNA-1205Lu cells developed proximal metastases (Figure 2c), which were remarkably smaller than those of the CTRL-shRNA-1205Lu challenged group. Examination of the lung showed high incidence of distal metastases foci in CTRL-shRNA group (10/10), whereas none of the mice in the TGFBI-shRNA group developed distal metastases (Figure 2c). In line, the mitotic figures were more abundant in CTRL-shRNA compared with the TGFBI-shRNA group (Figure 2d). Examination of draining and non-draining lymph nodes as well as several other organs (data not shown) did not reveal any other metastases development both in CTRL or TGFBI knockdown tumor-challenged mice. Immunohistochemical analyses showed intense TGFBI staining around infiltrating tumor cells in proximal and distal lung and ovary metastases in CTRL-shRNA-1205Lu-induced tumors (Figure 2e). In contrast, TGFBI-shRNA-1205Lu-induced tumors showed either faint TGFBI staining of the proximal metastasis and no or only background staining level of lung and ovary tissues. Thus, TGFBI is critical for distal metastases development in melanomas.

TGFBI knockdown does not interfere with melanoma cells' dissemination to the lung

We then tested whether the absence of TGFBI impairs the dissemination of melanoma cells through a kinetic study of micro-metastases formation in the lung. Mice received intravenous injection of CTRL- or TGFBI-shRNA-1205Lu cells at day 0. Groups of five mice were euthanized at different time points, and the lungs were analyzed for micro-metastases development. We took advantage of GFP expression (Supplementary Figure S1a online) to analyze the whole-lung cell suspensions by flow cytometry. Although, we did not reach significance, extravasation to the lung was detected as early as 24 hours post challenge with TGFBI-shRNA-1205Lu cells (mean of 0.02% GFP^+ cells in total lung) while it was detected only on day 3 with CTRL-shRNA-1205Lu cells (Figure 3a).

The percentage of CTRL-shRNA-1205Lu extravasating cells at day 5 remained similar to that at day 3, but a considerable decline in the percentage (from mean of 0.08 to 0.037%) of TGFBI-shRNA-1205Lu extravasating cells was observed after 5 days, and we could barely detect any GFP^+ cells after 7 days. On the contrary, increasing numbers of GFP^+ CTRL-shRNA-1205Lu cells were detected over time, reaching a mean of 0.4% by day 9. High-resolution two-photon microscopy analyzing the localization of melanoma cells to the lung corroborated with flow cytometry analysis (Figure 3b). Hematological dissemination of melanoma cells to the lung was

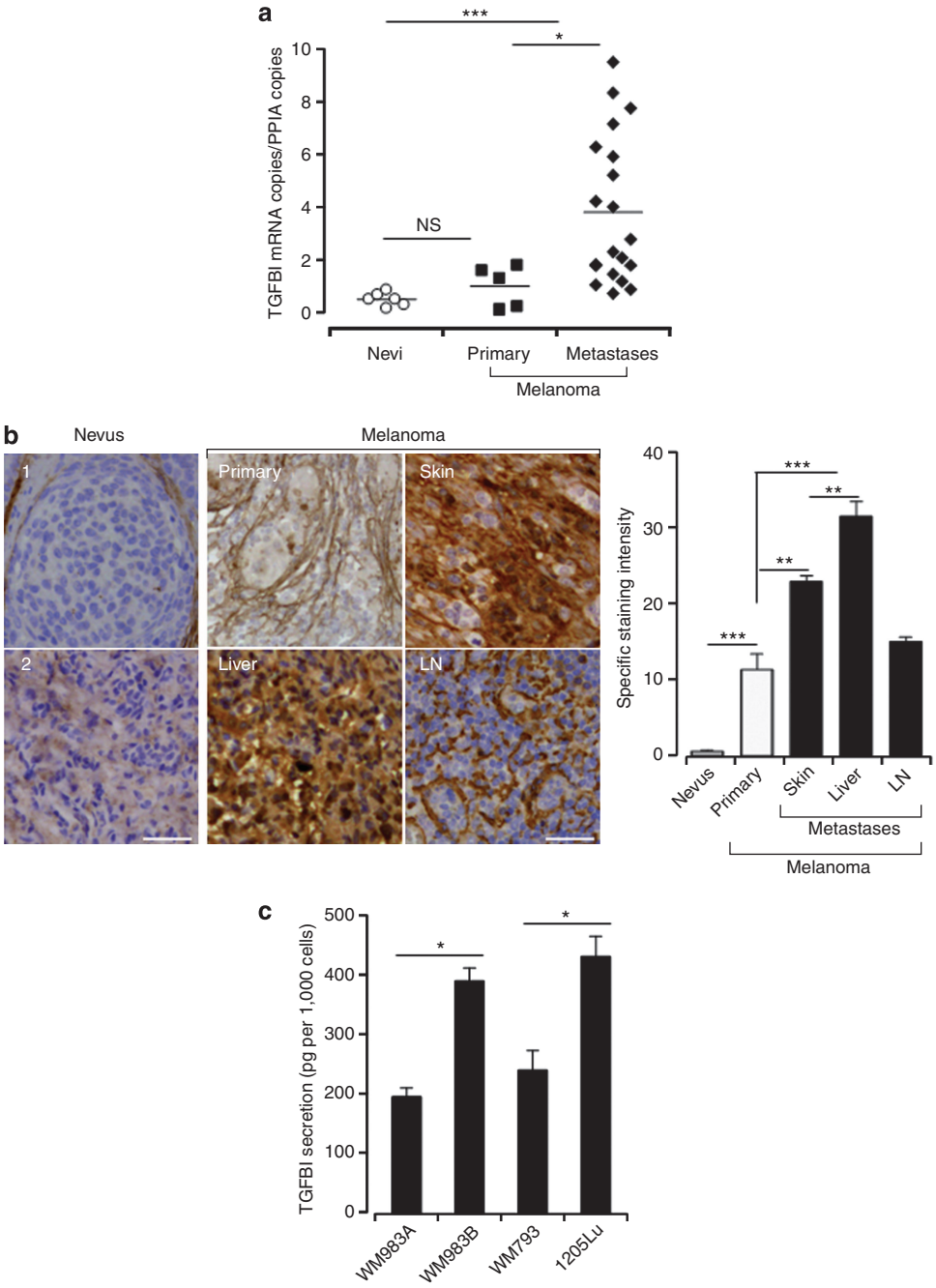

Figure 1. Human melanoma produces large amounts of transforming growth factor beta (TGFβ)-induced protein (TGFBI). (**a**) Expression of *TGFBI* mRNA relative to *PPIA* housekeeping gene mRNA in benign nevi, primary skin, and metastatic melanomas. Median values are 0.50 ± 0.087 (nevi), 1.15 ± 0.28 (primary skin), and 3.82 ± 0.28 (metastatic melanoma). (**b**) Representative images of TGFBI staining and quantification (right panel) in nine nevi (1 and 2), five primary skin melanoma (primary), and in five skin, five liver, five lymph node (LN) metastases. Bar = 50 μm. Mean values ± SEM are presented. (**c**) Secretion of TGFBI by primary (WM293A, WM793) and their respective metastatic (WM983B, 1205Lu) melanoma cell lines. Results are expressed as mean values ± SD of TGFBI (pg) secreted by 1,000 cells from three independent experiments performed in triplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$. NS, not significant.

clearly visible as single-cell micro-metastasis 5 days after implantation with both melanoma cell lines. However, by 9 days post implantation, several foci of larger metastases were detected only in the CTRL-shRNA-1205Lu group, whereas almost no GFP^+ cells were present in the TGFBI-shRNA group (Figure 3b).

Therefore, we investigated the role of TGFBI knockdown in cell migration, invasion, extravasation, and adhesion capacity *in vitro*. We found that TGFBI-shRNA-1205Lu cells not only have higher migration than CTRL-shRNA-1205Lu or wild-type (WT) 1205Lu cells (3.5-fold increase) (Supplementary Figure S2a online) but also higher capacity

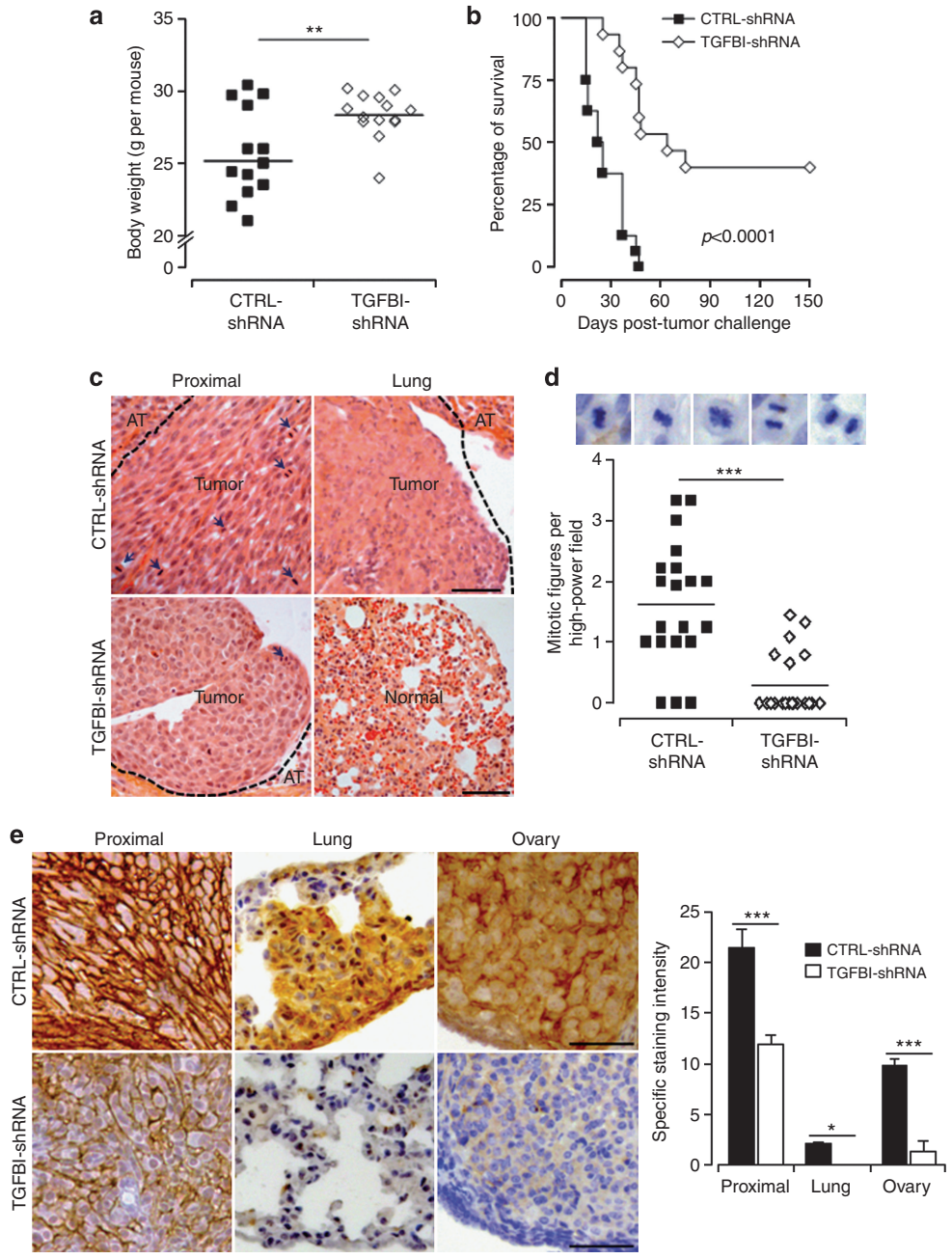

Figure 2. Transforming growth factor beta (TGFβ)-induced protein (TGFBI) expression is required for metastatic outgrowth. Nude mice received either CTRL- or TGFBI-shRNA-1205Lu melanoma cells. (**a**) Body weight analysis at 3 weeks post-tumor inoculation. (**b**) Kaplan–Meier disease-free survival curves. (**c**) Hematoxylin and eosin (H&E) staining of proximal and distal tissues indicating metastases formation (Tumor) and normal adjacent tissue (AT). Dashed lines delineate tumor tissue border from normal AT. Bar = 50 μm. Arrowheads point to mitotic figures. (**d**) H&E staining and quantification of frequently observed mitotic figures in proximal metastases taken with × 400 objectives. Each data point represents the average count of mitotic figures in 10 fields. Median (horizontal line) is shown. (**e**) Representative TGFBI expression (left panel) and quantification (right panel) in different tissues (× 200 magnification). Mean values ± SEM are presented. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

to invade (2.5-fold increase) (Figure 3c left panel and Supplementary Figure S2b online) and extravasate through endothelial cells (2-fold increase) (Supplementary Figure S2c online). TGFBI-shRNA-1205Lu cells also displayed higher capacity to adhere than WT and CTRL-shRNA-1205Lu cells (1.5-fold increase) (Figure 3c right panel and Supplementary Figure S2d). The addition of recombinant TGFBI restored invasion and adhesion to the level of WT cells (Figure 3c and Supplementary Figure S3 online), indicating that the observed increases are linked to the knockdown of TGFBI secretion in TGFBI-shRNA-1205Lu cells. Similar results were obtained when assays were conducted with cells engineered with a second TGFBI-shRNA (Supplementary Figures S2 and S3 online).

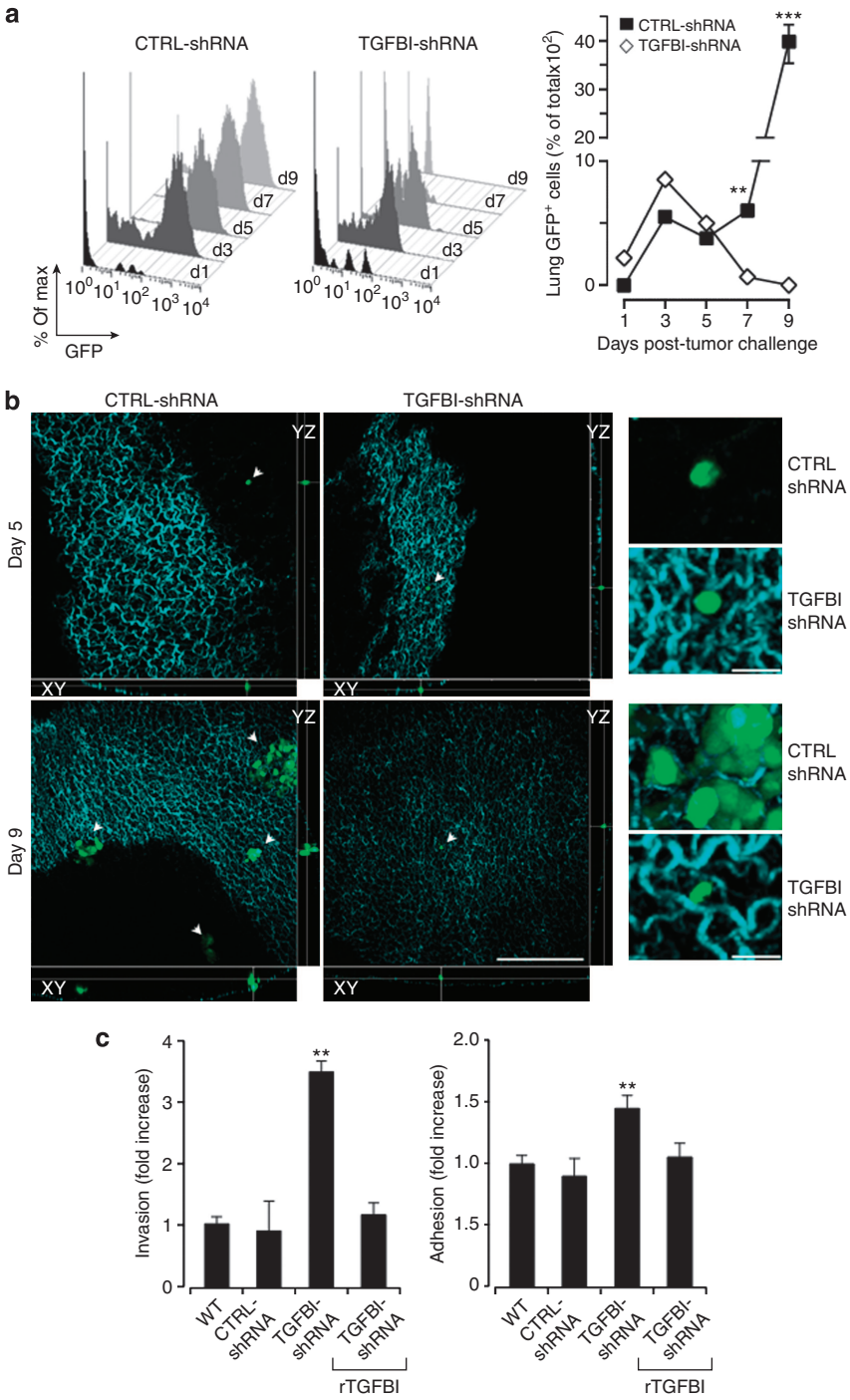

Figure 3. Transforming growth factor beta (TGFβ)-induced protein (TGFBI) silencing does not affect dissemination capacity of melanoma cells. (**a**) GFP^+ (green fluorescent protein–positive) cells detected in total lung from CTRL- or TGFBI-shRNA-1205Lu cells nude mice. Representative overlay of FACS plots at different time points (left panel) and quantification of GFP^+ cells given as mean values ± SEM (right panel). (**b**) Images on fixed lungs from maximum intensity projection of *z*-stacks (15 μm). YZ and XY orthogonal views are shown. Second-harmonic generation of type I collagen (cyan); GFP^+ CTRL- or TGFBI-shRNA-1205Lu cells (green). Arrowheads indicate GFP^+ cells. Bar = 100 μm. Higher magnification images are given, bar = 20 μm. (**c**) Wild-type (WT) or CTRL- or TGFBI-shRNA-1205Lu cells' invasion and adhesion presented as mean values ± SD from three independent experiments performed in triplicates. $^{**}P<0.01$, $^{***}P<0.001$.

Collectively, these *in vivo* and *in vitro* findings show that TGFBI-shRNA-1205Lu cells can extravasate like CTRL-shRNA-1205Lu cells, but their survival is impeded in a new microenvironment.

TGFBI is required for melanoma cells' proliferation and survival

We then investigated the role of TGFBI in tumor cell engraftment, namely cell growth and survival. Immunohistochemical analyses showed that while CTRL-shRNA-induced

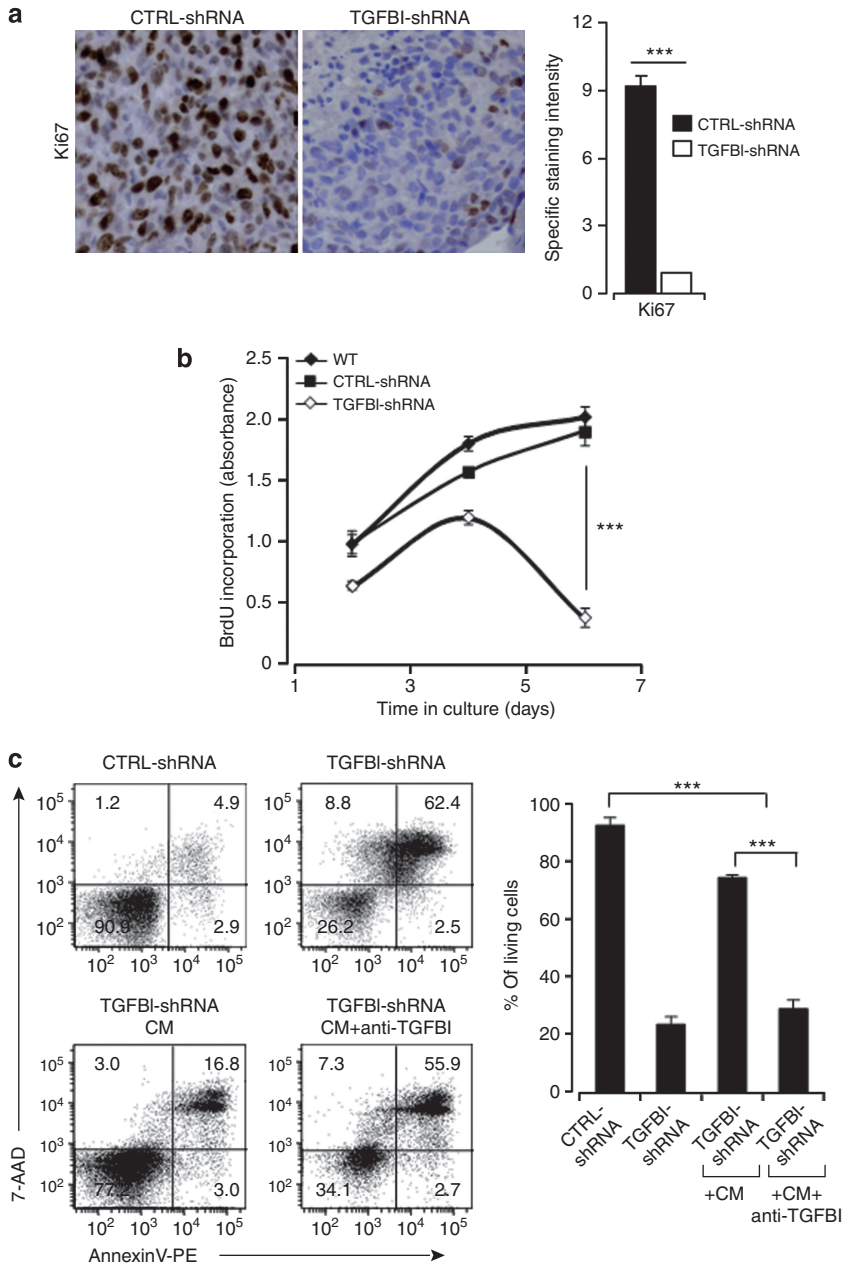

Figure 4. Transforming growth factor beta (TGFβ)-induced protein (TGFBI)-shRNA-1205Lu cells display lower proliferation rate and decreased survival. (**a**) Representative images (left panel) and quantification (right panel) of Ki67 expression in CTRL- ($n=10$) and TGFBI-shRNA-1205Lu ($n=9$) proximal tumors performed using 3DHISTECH panoramic slide viewer. Bar = 50 μm. (**b**) Proliferation of wild-type (WT), CTRL- and TGFBI-shRNA-1205Lu cells was determined at indicated time points. Results are presented as mean values ± SD of absorbance from three independent experiments performed in triplicates. (**c**) Apoptosis and survival of TGFBI-shRNA-1205Lu cells. Conditioned medium (CM) from CTRL-shRNA-1205Lu or anti-TGFBI-blocking antibody was added. Representative dot-plot showing the percentage of cells in each quatrain (left panel), and the percentage of 7-AAD^{neg}Annexin^{neg} cells presented as mean values ± SD from three independent experiments (right panel). ***$P<0.001$. BrdU, 5-bromo-2′-deoxy-uridine; PE, phycoerythrin; 7-AAD, 7-aminoactinomycin D.

tumors display intense staining of cellular proliferation marker Ki67, TGFBI-shRNA-induced tumors display reduced numbers of Ki67$^+$-proliferating cells (Figure 4a). Accordingly, we evaluated the effects of TGFBI knockdown on melanoma cells growth *in vitro*. Proliferation of TGFBI-shRNA-1205Lu cells was slower compared with WT and CTRL-shRNA-1205Lu cells (Figure 4b). Notably TGFBI-shRNA-1205Lu cells showed a drastic decline in their growth beginning at day 4 and demonstrated the lowest rate of proliferation by day 6 (10-folds less than WT and CTRL-shRNA-1205Lu cells). Melanoma cells engineered with another TGFBI-shRNA displayed similar proliferation profile (Supplementary Figure S2e online). 7-Aminoactinomycin D (7-AAD)/Annexin-V staining showed that only 20% of TGFBI-shRNA-1205Lu cells survived after 4 days of culture compared with 90% of CTRL-shRNA-1205Lu cells (Figure 4c). Culturing TGFBI-shRNA-1205Lu cells in the

presence of conditioned medium from CTRL-shRNA-1205Lu cultures increased the percentage of living TGFBI-shRNA-1205Lu cells to 70%, and the presence of a blocking anti-TGFBI antibody completely abrogated this rescue (Figure 4c). Thus, secretion of TGFBI contributes to melanoma cells' growth and survival within their microenvironment.

Melanoma cells lacking TGFBI have defective cell cycle progression

As the successful division and replication of cells is essential for their survival, we examined the progression in cell cycle of CTRL-shRNA-1205Lu cells in comparison to TGFBI-shRNA-1205Lu over 5 days (Figure 5a and b). On day 1 TGFBI-shRNA-1205Lu cells tend to increase their G0/G1 phase ($P=0.08$) and significantly decreased G2/M phases populations ($P=0.03$) compared with CTRL-shRNA-1205Lu cells. On day 2, TGFBI-shRNA-1205Lu cells showed significantly decreased G0/G1 and G2/M phases ($P=0.01$ and $P=0.006$, respectively) compared with CTRL-shRNA-1205Lu cells, whereas the percentage of cells in S phase increased without reaching significance. This is likely due to the emergence of a subG1 phase population (15% apoptotic cells), while CTRL-shRNA-1205Lu cells did not show any apoptotic cells on day 2. At the end of the culture, G0/G1, S, and G2/M phase populations presented, respectively, nearly 71, 13, and 10% in CTRL-shRNA-1205Lu cultures compared with 44, 5 and 3% in TGFBI-shRNA-1205Lu cultures ($P=0.0001$, $P=0.007$, and $P=0.02$, respectively). Nearly 6% of cell death was observed in CTRL-shRNA-1205Lu cultures compared with 48% in TGFBI-shRNA-1205Lu cultures. These results indicate that TGFBI-shRNA-1205Lu cells have disturbed cell cycle progression likely with defects in G1/S transition and S phase regulation. Therefore, we analyzed the expression of the cyclin-dependent kinases (CDKs) and their regulators the cyclins (CCN). Quantitative reverse transcriptase–PCR (RT-PCR) analysis showed that TGFBI-shRNA-1205Lu cells express extremely low levels of CDK2 and significantly lower level of CDK6 compared with CTRL-shRNA-1205Lu cells (Figure 5c). TGFBI-shRNA-1205Lu cells also expressed very low levels of both CCNA and CCNE compared with CTRL-shRNA-1205Lu cells (Figure 5d). CCNE/CDK2 complex activity is required for G1/S transition. CCNA regulates two different phases, S and M, in function of whether it binds to CDK2 or CDK1, respectively. CDK6 is a catalytic subunit of the complex CCND1/CDK6 that is important for cell cycle G1 phase progression and G1/S transition.

Together, these results indicate that the secretion of TGFBI by melanoma cells is likely implicated in maintaining the expression of key regulators of their cell cycle and in the regulation of melanoma metastatic outgrowth.

DISCUSSION

Through a knockdown approach and *in vivo* metastasis model, we demonstrate the implication of TGFBI in melanoma distal metastasis formation through regulation of melanoma cells growth.

We found that TGFBI is expressed in nevus and melanoma clinical specimens. However, it was more abundant, although with a scattered pattern, in metastatic melanoma tissues. Metastatic melanoma cell lines also secreted higher amounts of TGFBI compared with their primary counterparts. Thus the expression of TGFBI in melanoma is proportional to tumor progression stage and aggressiveness. Melanoma tumors are marked by their extensive heterogeneity (Fidler and Kripke, 1977; Banerjee and Harris, 2000), and specific transcriptional signatures delineate various melanoma cells subpopulations (Haqq *et al.*, 2005; Hoek *et al.*, 2006). *In vivo,* these specific transcriptional signatures are linked and reversible given appropriate signals and microenvironment cues, suggesting that melanoma progression is associated with transcription signature plasticity (Hoek *et al.*, 2008). Heterogeneity and plasticity could explain the scattered pattern of TGFBI by different metastatic melanoma clinical specimens. In this perspective, our findings endorse the expression of TGFBI by melanoma as part of their plasticity towards aggressive metastatic phenotype. Our *in vivo* studies in a model of metastasis strongly support this notion. We found that the lack of TGFBI not only reduces tumor incidence but also distant metastases. That the secretion of TGFBI by melanoma cells promotes their development and permits their metastatic outgrowth suggests that TGFBI is part of the tumor-induced ECM modifications allowing progression and dissemination.

In ovarian cancer, TGFBI production by peritoneal cells promotes motility/invasion and adhesion of cancer cells to peritoneal cells increasing their metastatic potential (Ween *et al.*, 2011). Human colon cancer cells lacking TGFBI have decreased capacity to extravasate and decreased metastatic potential (Ma *et al.*, 2008). In our melanoma model, TGFBI knockdown increases the capacity of tumor cells to migrate/invade/extravasate and does not affect their dissemination capacity *in vivo*. This is in line with TGFBI being an anti-adhesive protein for melanoma cells (Nummela *et al.*, 2012) and indicates that TGFBI in melanoma potentiates metastases in a manner distinct from that operating in other tumors. One possible mechanism by which TGFBI affects cell adhesion and migration is through its negative effect on cytoskeleton dynamics. Downregulation of TGBFI in melanoma cells results in increased co-localization of actin filaments, β1 integrins, and talin (Nummela *et al.*, 2012). TGFBI also stabilizes microtubules (Ahmed *et al.*, 2007), which could also affect the migration process, and TGFBI silencing would favor microtubule dynamics and thereby cell motility and invasion. The lack of TGFBI resulted in a drastic decrease in the capacity of melanoma cells to proliferate to form micro-metastases. In line, we show that TGFBI produced from melanoma cells is critical for their proliferation, and their growth inhibition and defects in cell cycle progression were concomitant with drastic cell death. This finding suggests that the drastic decrease in cell proliferation observed *in vivo* is likely due to extensive cell death in the absence of secreted TGFBI. This indicates that even if melanoma cells lacking TGFBI have increased motility/invasion/extravasation, their establishment, survival, and growth within a new microenvironment, which is part of the overall metastatic process, is impaired.

If TGFBI produced from melanoma cells is functionally linked to their own growth and survival in distal metastases,

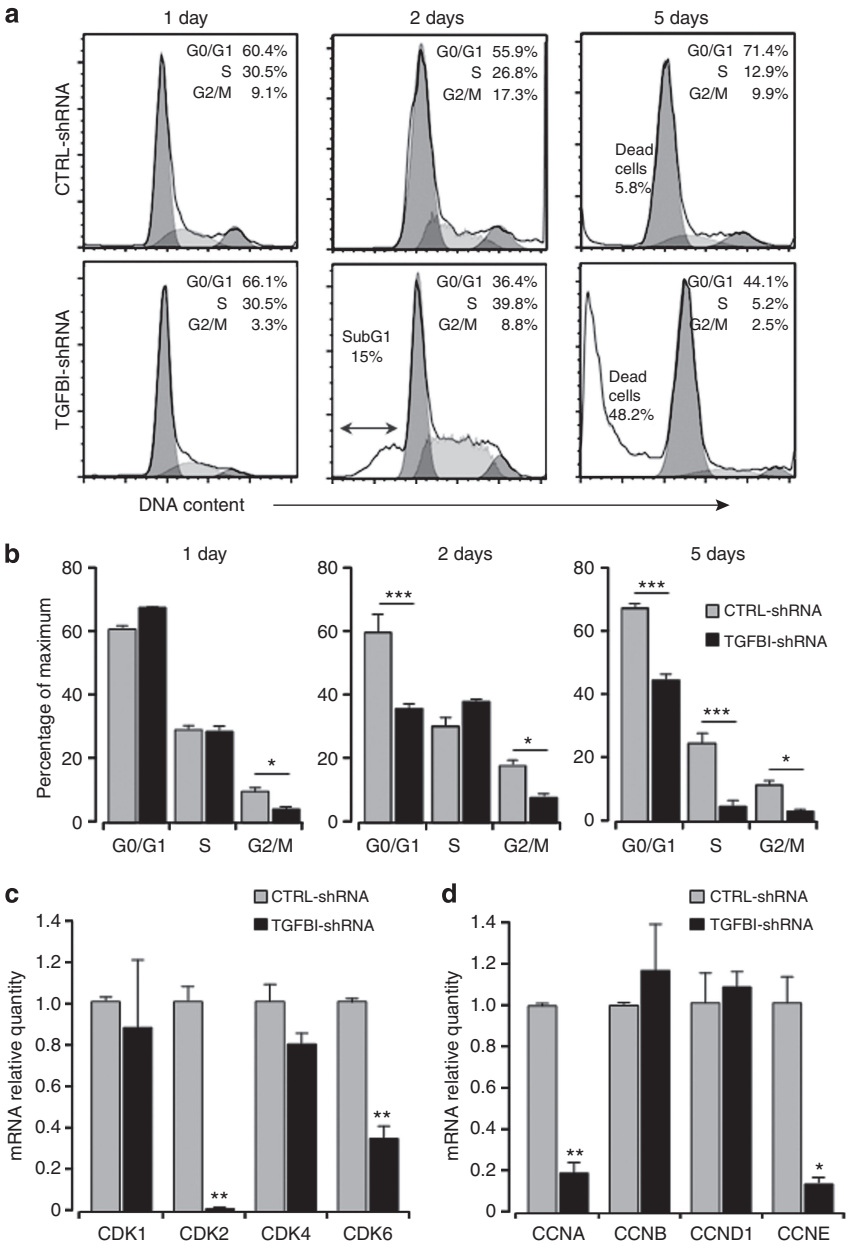

Figure 5. Transforming growth factor beta (TGFβ)-induced protein (TGFBI)-shRNA-1205Lu cells have defective cell cycle progression. (**a**) Cell cycle distribution of CTRL- and TGFBI-shRNA-1205Lu cells at indicated time points. The percentage of cells in each phase of the cycle is shown for each panel. Percentages of sub-G1 and dead cells are given. (**b**) Quantification given as percentage of maximum calculated from replicate experiments. (**c** and **d**) mRNA levels for cell cycle regulatory cyclin-dependent kinases (CDK1, 2, 4, and 6) (**c**) and cyclins (CCNA/B, D1, and E) (**d**) genes were determined for CTRL- and TGFBI-shRNA-1205Lu cells. Results from three independent experiments are expressed as mean values of relative mRNA quantity of each CDK or CCN against *RPL13* gene. Error bars are ± SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ comparing TGFBI- with CTRL-shRNA-1205Lu cells.

how does it operate? Our rescue experiments in which shRNA effect was reversed by exogenous TGFBI in conditioned medium or recombinant TGFBI indicate that TGFBI released by melanoma cells, rather than an intracellular form, likely regulates their proliferation and survival. Actions of secreted TGFBI depend on interactions between other ECM proteins and specific integrins present in tumor microenvironment. Growth and survival of melanoma cells through integrins is regulated by the composition and the physical nature of the ECM proteins (Petitclerc *et al.*, 1999; Henriet *et al.*, 2000). Beside tumor-derived proteases, tumor-secreted ECM proteins can also have critical roles in ECM modification/re-organization to promote growth and survival (Berking *et al.*, 2001; Botti *et al.*, 2012). Thus TGFBI upon its secretion by melanoma cells could also be an actor of ECM remodeling and re-organization providing a supporting scaffolding and a positive feedback stimulation of tumor growth/survival. This could be supported by the capacity of TGFBI to bind to several

ECM proteins, including collagens, fibronectin, proteoglycans, and periostin (Thapa *et al.*, 2007), and its localization within fibrillar fibronectin structures in melanoma tumors (Nummela *et al.*, 2012).

TGFBI knockdown induced growth inhibition through defects in G1/S transition and S phase regulation by controlling the expression of CDK2 and CDK6 and their regulators CCNA and CCNE. Regulation of CCNs requires coordinated signaling from soluble factors and ECM/integrins. The signaling pathways activated upon TGFBI/integrins interactions are not yet fully determined, and we did not investigate the signaling pathways supported by TGFBI in melanoma or the implicated integrin. However, as part of intracellular signaling, TGFBI interaction with integrins supports actin stress fiber formation in adhering cells (Billings *et al.*, 2002), phosphorylation of AKT, extracellular signal–regulated kinase, focal adhesion kinase, and paxillin in U87 and vascular smooth cells (Kim *et al.*, 2003; Lee *et al.*, 2006), and activation of phosphatidylinositol 3′-kinase pathway in keratinocytes (Oh *et al.*, 2005).

TGFBI was originally identified as a TGFβ-inducible gene (Thapa *et al.*, 2007). As increased TGFβ expression was correlated with melanoma progression and implicated in promoting aggressiveness and metastasis (Javelaud *et al.*, 2008), it is possible that TGFBI acts among the effectors in mediating the prometastatic/metastatic activity of TGFβ in these tumors. In breast cancer, a link between TGFβ signaling, TGFBI expression, and metastasis was proposed (Shipitsin *et al.*, 2007). In human melanomas, two populations with very distinct gene expression profiles were identified: neural crest and melanocytic differentiation phenotype and more aggressive phenotype displaying a TGFβ signature (Hoek *et al.*, 2006; Hoek, 2007; Hoek *et al.*, 2008). TGFβ has anti-proliferative effects on normal melanocytes, whereas melanoma cells display increased resistance to TGFβ-dependent growth inhibition, proportional to tumor progression stage (Krasagakis *et al.*, 1998) and is probably highly specific to cell cycle progression (Rodeck *et al.*, 1999; Reed *et al.*, 2001; Poser *et al.*, 2005). Our results demonstrate that the lack of TGFBI decreases the expression of CDK2 and CCNE, thus CDK2/CCNE complex, which binds to p21 inhibiting its activity and promoting cell cycle progression. Therefore, we propose the abundance of TGFBI in metastatic melanoma among the mechanisms mediating the desensitization of aggressive melanoma cells to TGFβ anti-proliferative effect and suggest a link between TGFβ signaling, TGFBI expression, and melanoma metastatic growth.

In summary, we describe a mechanism of melanoma metastatic outgrowth via promotion of growth/survival by TGFBI. The management of melanoma metastatic outgrowth remains a clinical challenge, and elevated secretion of TGFBI could be used as a biomarker for indicating patients with progressing tumors. Although this warrant further investigations, TGFBI being an ECM protein, peptides or antibodies that inhibit or limit its action might be among the strategies to control or limit melanoma metastases.

MATERIALS AND METHODS

Patient samples and melanoma cell cultures

Frozen human nevus ($n=6$), primary skin melanoma ($n=5$), and metastatic melanoma ($n=20$) specimens (Saint Louis Hospital, Dermatology Department, Paris, France) were used for RNA extraction. Paraffin blocks of human nevus ($n=9$), primary skin melanoma (5), and skin ($n=5$), lymph node ($n=5$), and liver ($n=5$) metastatic melanomas were obtained from the laboratory of anatomy and pathological cytology (Purpan Hospital, Toulouse, France). Specimens were collected, and the study was performed according to the guidelines of Hospitals institutional boards and Ethic Committees after written informed patients' consent, in accordance with the Helsinki Declaration. Human melanoma cell lines (gift from Alain Mauviel (Curie Institute, Orsay, France)) derived from tumor biopsies from patients with primary (WM983A and WM793) or metastatic (WM983B and 1205Lu) melanoma lesions (Alexaki *et al.*, 2010) were grown in DMEM/F-12 medium supplemented with 10% fetal calf serum (Invitrogen, Saint Aubin, France).

Stable TGFBI silencing in 1205Lu human melanoma cells

Stably transduced cells were generated by lentiviral infection using pLKO.1-puro-CMV-TurboGFP plasmid (Sigma-Aldrich, Saint-Quentin Fallavier, France) encoding non-targeting shRNA (CTRL-shRNA, Sigma-Aldrich SHC002V) or shRNA targeting the coding sequence of TGFBI (TGFBI-shRNA clone TRCN0000062174 and TRCN0000062175). RT-PCR and ELISA verified stable reduction of *TGFBI* mRNA expression and TGFBI secretion over time. Detailed procedures are described under Supplementary Information online.

Gene expression

Total mRNA was isolated using RNeasy kit (Qiagen, Valenica, CA) and reverse transcribed with Omniscript (Qiagen). cDNAs were then processed for RT-PCR using SYBR Green PCR kit (Applied Biosystems, Villebon-sur-Yvette, France). Gene-specific primers are summarized in Supplementary Table S1 online, and technical details are under Supplementary Information online.

Experimental metastasis in mice

Six to 8-week-old female nude mice (NMRI Nu/Nu) were injected into the retro-orbital venous sinus with 5×10^5 melanoma cells expressing CTRL-shRNA or TGFBI-shRNA as described (Galaup *et al.*, 2006). Mice were monitored twice a week to detect weakness, weight loss, or morbidity, and those losing $>15\%$ of their weight were euthanized. Spleen, lymph nodes, liver, lung, kidney, colon, ovary, and uterus were removed and processed for histopathological analysis as described (Chabot *et al.*, 2011). All animal experiments were performed in agreement with the European Union guidelines and approved by the local Ethic Committee (MP/02/37/06/08).

Histopathology and immunostaining

Formalin-fixed paraffin-embedded specimens of melanoma tissues were processed for hematoxylin/eosin staining or immunohistochemistry. Immunohistochemistry were performed with anti-TGFBI (Proteintech, Manchester, UK) or anti-Ki67 (Thermo Scientific, Courtaboeuf, France) mAbs, revealed using the ABC-peroxidase kit (Vector Laboratories, Peterborough, UK), analyzed with a Leica DMR microscope (Leica Microsystems, Nanterre, France) at $\times 200$

objective, and specific pixel intensities over field area were calculated in mm^2 using 3DHISTECH panoramic slide viewer (3DHISTECH Kft, Budapest, Hungary). Details are under Supplementary Information online.

Two-photon microscopy and second-harmonic generation

Lungs were removed, fixed in 3% formaldehyde, and tissues were mounted using surgical glue. Images were taken on a Zeiss inverted two-photon microscope ($\times 20$ water-immersion objective) (Carl Zeiss, Jena, Germany). Helical structures of collagen fibers were imaged using the second-harmonic generation structures. The appearance of lung metastases was followed using GFP. *Z*-stack images were collected over 250-μm deep sections.

Proliferation, apoptosis, and cell cycle assays

CTRL- and TGFBI-shRNA-1205Lu cells were grown as indicated, and proliferation, apoptosis, and cell cycle were analyzed. Proliferation was evaluated using 5-bromo-2′-deoxy-uridine labeling and detection kit III (Roche, Boulogne-Billancourt, France) by absorbance measurement at 405 nm. Apoptosis was determined using 7-AAD and Annexin-V–phycoerythrin (BD Biosciences, Le Pont-de-Claix, France) staining and flow cytometry (FACS Canto II) analysis (BD Biosciences). Supernatant from CTRL-shRNA-1205Lu cultures was used to replace that of TGFBI-shRNA-1205Lu cultures in the presence or absence of an anti-TGFBI-blocking antibody (Proteintech). Cell cycle was analyzed on fixed cells and stained using propidium iodide supplemented with RNAse A (Sigma-Aldrich). DNA contents were analyzed on a FACS Calibur cytometer (BD Biosciences). Percentages of cells in G0/G1, S, and G2/M phases were determined by Watson method using the FlowJo software (Celeza, Olten, Switzerland).

Migration, matrigel invasion, and extravasation assays

CTRL- and TGFBI-shRNA-1205Lu cells were harvested, re-suspended in serum-free medium (Miltenyi Biotech, Bergisch Gladbach, Germany) with or without soluble recombinant TGFBI (100 nM) (R&D Systems, Lille, France) and seeded at 5×10^4 cell on Transwell inserts coated or not with growth-factor reduced Matrigel (BD Bioscience). After 6 hours, migrating/invading cells were fixed, stained, and counted under a light microscope. Extravasation assays were conducted following the above procedure but using fluoroblok transwell inserts coated with matrigel and human microvascular endothelial cells seeded at 5×10^4 cell/well, and DiO-labeled extravasading melanoma cells were directly counted using fluorescence microscopy. Details are under Supplementary Information online.

Adhesion assays

CTRL- and TGFBI-shRNA-1205Lu melanoma cells were harvested, re-suspended in serum-free medium with 0.1% BSA, seeded in a 96-well plate at 2.5×10^4 cell/well, and allowed to adhere for 1 hour. Cells were then fixed with 1% formaldehyde, stained with violet cristal, then lysed with 1% SDS, and absorbance at 600 nm using a microplate reader determined the relative adherence.

Statistical analyses

For TGFBI gene expression, migration/invasion/extravasation, and proliferation assays, we used the *t* test to calculate 95% confidence intervals on differences between median or mean and Mann–Whitney test to obtain *P* value. Data related to experimental metastasis in mice were analyzed using two-way analysis of variance test. All statistical tests were two-sided. $P<0.05$ was considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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